Genomic Characterization of Recrudescent *Plasmodium malariae* after Treatment with Artemether/Lumefantrine

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Plasmodium malariae is the only human malaria parasite species with a 72-hour intraerythrocytic cycle and the ability to persist in the host for life. We present a case of a *P. malariae* infection with clinical recrudescence after directly observed administration of artemether/lumefantrine. By using whole-genome sequencing, we show that the initial infection was polyclonal and the recrudescent isolate was a single clone present at low density in the initial infection. Haplotypic analysis of the clones in the initial infection revealed that they were all closely related and were presumably recombinant progeny originating from the same infective mosquito bite. We review possible explanations for the *P. malariae* treatment failure and conclude that a 3-day artemether/lumefantrine regimen is suboptimal for this species because of its long asexual life cycle.

During the past decade, intensification of malaria control efforts has substantially reduced the global burden of malaria from *Plasmodium falciparum*. This trend has often been associated with increased recognition of the burden of malarial disease caused by the other *Plasmodium* species (*1*). *P. malariae,* 1 of the 6 *Plasmodium* species that commonly infect humans, is endemic throughout parts of Africa (*2*,*3*), South America (*4*), Asia, and the western Pacific (*5*). *P. malariae* is unique among the human-infective *Plasmodium* species in having a 72-hour intraerythrocytic life cycle with variable but often prolonged pre-erythrocytic intrahepatic development (*6*). *P. malariae* can persist in the human host for years and possibly an entire lifetime. Although it is often asymptomatic, chronic parasitemia in endemic areas is associated with substantial rates of illness, including anemia and nephrotic syndrome (*7*–*9*).

A key strategy for malaria elimination is strengthening of health systems to deliver early diagnosis and highly effective therapy. Artemisinin-based combination therapy (ACT) has been central to this approach, with proven efficacy against multidrug-resistant *P. falciparum*, multidrug-resistant *P. vivax,* and *P. knowlesi* (*10*–*13*). In recent years, there have been increasing calls for a universal policy of ACT for all species of malaria (*10*–*13*). However, the efficacy of ACT against *P. malariae* is poorly documented.

Although chronic infection with *P. malariae* is well-recognized (*14*), little is known regarding how the parasites manage to evade host immunity and the intrahost dynamics of the underlying parasite population. Recent advances in molecular genetics have produced the first descriptive analyses of the whole genome sequence of *P. malariae* (*15*,*16*). The *P. malariae* reference genome is 33.6 Mb in size, has 6,540 genes, and has an average guanine plus cytosine content of 24% (*15*).

We report a case of a *P. malariae* infection in a patient residing in a non–malaria-endemic environment that resulted in recrudescence months after treatment with artemether/lumefantrine (AL). By using whole-genome sequencing of isolates from the initial and the recrudescent infections, we show that the 2 major *P. malariae* haplotypes, constituting ≈90% of the parasite load in the initial infection, were cleared successfully by AL, whereas a third haplotype, constituting a minority subpopulation in the initial infection, survived and recrudesced.

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Results

The Patient

A 31-year-old Uganda-born man, weighing ≈77 kg (≈170 lbs), who had been a resident in Australia for 5 years sought care at Royal Darwin Hospital (Darwin, Northern Territory, Australia) on March 1, 2015, with a 4-day history of fevers and headaches. He had returned to Australia 56 days previously after a 2-week trip to Uganda visiting friends and relatives (Figure 1, panels A, E). He had spent 14 days in a rural malaria-endemic area in eastern Uganda. Although he had not taken regular malaria prophylaxis, he had self-medicated with a locally acquired oral course of AL on the second and third days of his trip, despite being clinically well (Figure 1, panels B, D). He returned to Australia (now a malaria-free country) in January 2015 until seeking care after a short febrile illness in late February. On examination, he had a tympanic temperature of 37.5°C and a heart rate of 110 beats/min but no manifestations of severe malaria. Rapid diagnostic testing with BinaxNOW (Binax, Inc. Inverness Medical Professional Diagnostics, Scarborough, ME, USA) for malaria was positive for aldolase but negative for histidine-rich protein 2. Species-specific PCR was positive for *P. malariae* and negative for all other *Plasmodium* species (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/8/16-1582- Techapp1.pdf). Thick and thin blood film examination confirmed *P. malariae* parasitemia (12,140 parasites/μL) with all stages of asexual development visible on the blood film (online Technical Appendix Figure 1, panels A, B). The blood film was otherwise unremarkable; in particular, no evidence for hyposplenism was found. The patient was not immunosuppressed, and an HIV serologic test was negative. A hepatitis C

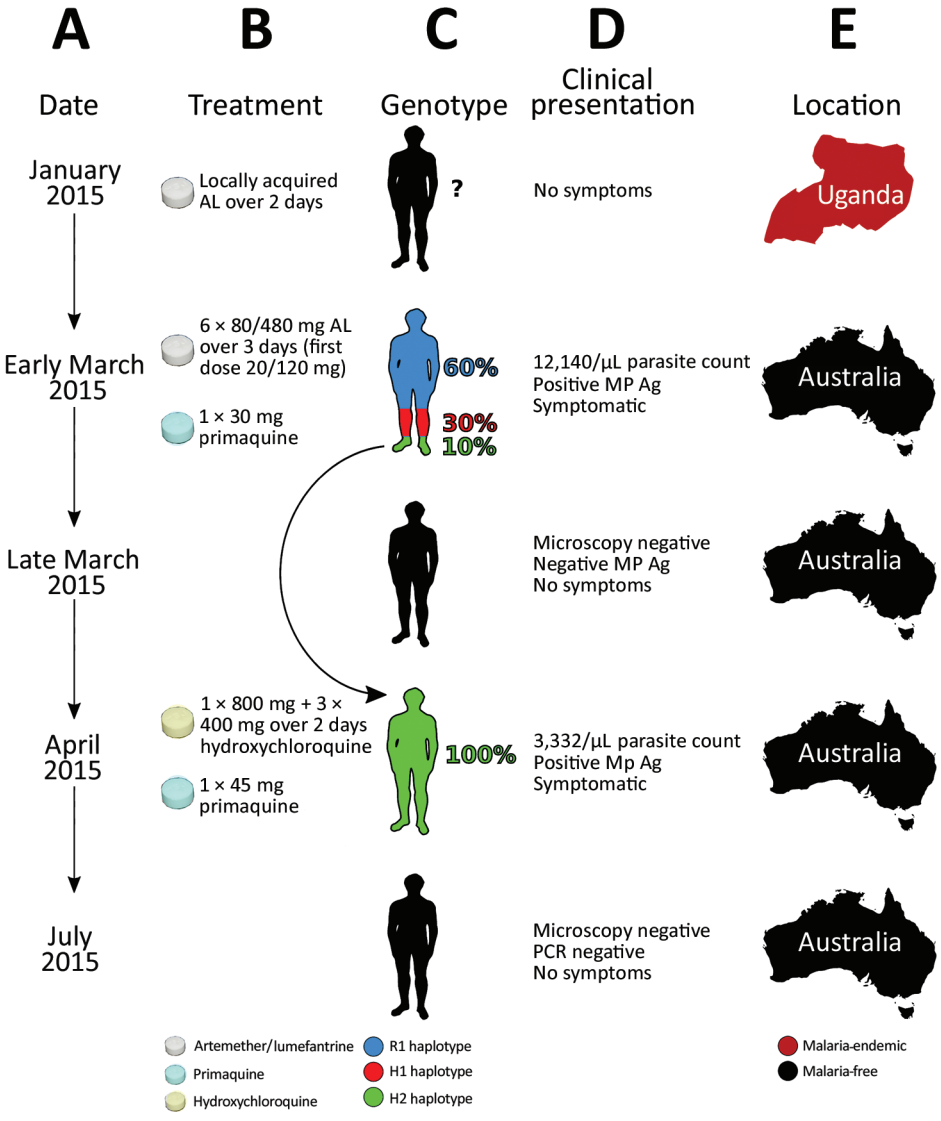

Figure 1. Timeline of the clinical case of a patient with *Plasmodium malariae* infection diagnosed and treated at Royal Darwin Hospital, Darwin, Northern Territory, Australia, March–April 2015, showing the timing (A), treatment (B), parasite's genotype as inferred from whole-genome sequencing (C), clinical presentation (D), and location (E). The rounded arrow indicates the recrudescence of the minor haplotype 2 in the initial infection to dominate monoclonally in the second infection. AL, artemether/lumefantrine; H1, haplotype 1; H2; haplotype 2; MP Ag, pan-malarial antigen; R1, reference haplotype.

serologic test was positive but with a viral load that was below the limit of quantification (<12 IU/mL).

The patient was administered a single 20/120 mg tablet of AL on the first day because of a prescribing error but subsequently continued with a supervised standard regimen of 80/480 mg every 12 hours taken with fatty food to complete a full course of 6 doses over 3 days, equivalent to a total dosage of 6.2 mg/kg of artemether and 37.4 mg/kg of lumefantrine. Glucose 6-phosphate dehydrogenase function was normal, and a single 30-mg dose of primaquine was administered on day 2. His hemoglobin was 126 g/dL and he received no blood transfusion. After treatment, his parasitemia declined to 1,269/μL at 32 hours, 488/μL at 41 hours, and 55/μL at 56 hours. He was afebrile and symptom-free within 36 hours of admission. However, before discharge on day 6, thick blood film examination was still positive (192/μL), but by day 11, his repeat blood film examination and his aldolase rapid diagnostic test results were negative.

The patient remained in urban Darwin but returned to the hospital 52 days later, on April 22, 2015, with a 2-week history of fevers, fatigue, and headache. Microscopy again identified *P. malariae* with a parasite count of 3,332/μL (online Technical Appendix Figure 1, panels C, D). Chloroquine was unavailable, so the patient was re-treated with oral hydroxychloroquine with an 800-mg loading dose, followed by 400 mg at 6 hours, 400 mg at 24 hours, 400 mg at 48 hours, and a single 45-mg dose of oral primaquine. The parasite count declined rapidly to 37/μL at 28 hours, 191/μL at 49 hours, and 76/μL at 88 hours of treatment. His symptoms resolved rapidly. Thick and thin blood films were negative on day 4 and remained negative on retesting at days 8, 35, 41, and 84, and the patient remained free of symptoms throughout. A PCR on blood collected at 12 weeks was also negative.

Whole-Genome Sequencing

Extensive sequencing was performed from blood samples obtained from the initial (PmUG01) and recrudescent (PmUG02) infection (online Technical Appendix Table 1), covering >99% of the genome at >20× for both infections. By using additional *P. malariae* samples published previously (*15*), we identified single-nucleotide polymorphisms (SNPs) using GATK's UnifiedGenotyper (Broad Institute, Cambridge, MA, USA) (*17*) and filtered them based on several parameters (online Technical Appendix Table 2). A multidimensional scaling plot of the samples based on their SNP allele frequency-spectra revealed that PmUG01 and PmUG02 were more closely related to each other than to any of the other samples (online Technical Appendix Figure 2), as expected if they were related recombinants derived from the same original infection.

Searching solely for SNPs that distinguish PmUG01 and PmUG02, we identified 2,631 variants after filtering (online Technical Appendix Table 2). PmUG01 was the sample from which the reference genome (R1) was constructed (*15*), and only 1 SNP in PmUG01 suggested a nucleotide base different from the reference strain, probably because it was in a repetitive region (online Technical Appendix Table 4). PmUG01 appeared to be a polyclonal infection with a bimodal distribution of alternate (i.e., nonreference) alleles at frequencies of 0.15 and 0.35 (online Technical Appendix Figure 3, panel A). Conversely, PmUG02 appeared to be a monoclonal infection with ≈85% of sites being either fixed for the reference allele or for an alternative allele (online Technical Appendix Figure 3, panel B). Comparison of the initial and recrudescent infections revealed that heterozygous sites in the initial infection had become either homozygous alternate (≈40%) or homozygous reference (≈45%) (online Technical Appendix Table 4). Analysis of the genotype calls across the genome (online Technical Appendix Figure 4) revealed that, whereas the heterozygous sites from the initial infection were spread evenly across the 14 chromosomes, the homozygous alternate sites in the recrudescent infection were present in distinct clusters, implying that the initial infection was polyclonal and that the recrudescence was attributable to a single clone that was closely related to the reference clone.

Comparison of the distribution of the alternate allele frequencies throughout the genome of the initial and recrudescent strains (Figure 2) revealed bands of alleles at frequencies of ≈0.15 and ≈0.35 in the initial infection spatially clustered throughout the genome. The alleles that increased in relative frequency in PmUG02 were mostly at frequencies of ≈0.15, whereas the alleles at frequencies of ≈0.35 decreased in frequency and the positions became homozygous reference in PmUG02 (Figure 2). These data strongly suggested that, in addition to R1, 2 minor clones (minor haplotypes) were also present. Of these 2, the clone with the haplotype comprising alternate alleles at frequencies of ≈0.35 (H1) appeared to have been eliminated during the drug treatment because no alleles specific to H1 were present in the recrudescent infection. The other minor clone comprised a haplotype with alternate alleles at frequencies of ≈0.15 (H2) in the initial infection; this clone appeared to have caused the recrudescence (Figure 1, panel C). Based on the relative alternate allele frequencies of the 3 haplotypes in the initial infection, ≈60% of the parasites were of the R1 haplotype, 30% of H1, and 10% of H2. These estimates were broadly consistent with the ratio of alleles in tri-allelic sites (0.69:0.22:0.09) (online Technical Appendix Table 5). The ratio of alleles in these tri-allelic sites changes markedly in PmUG02 (0.13:0.06:0.81), with over half of sites becoming homozygous for H2 but with some heterogeneity in the other sites (online Technical Appendix

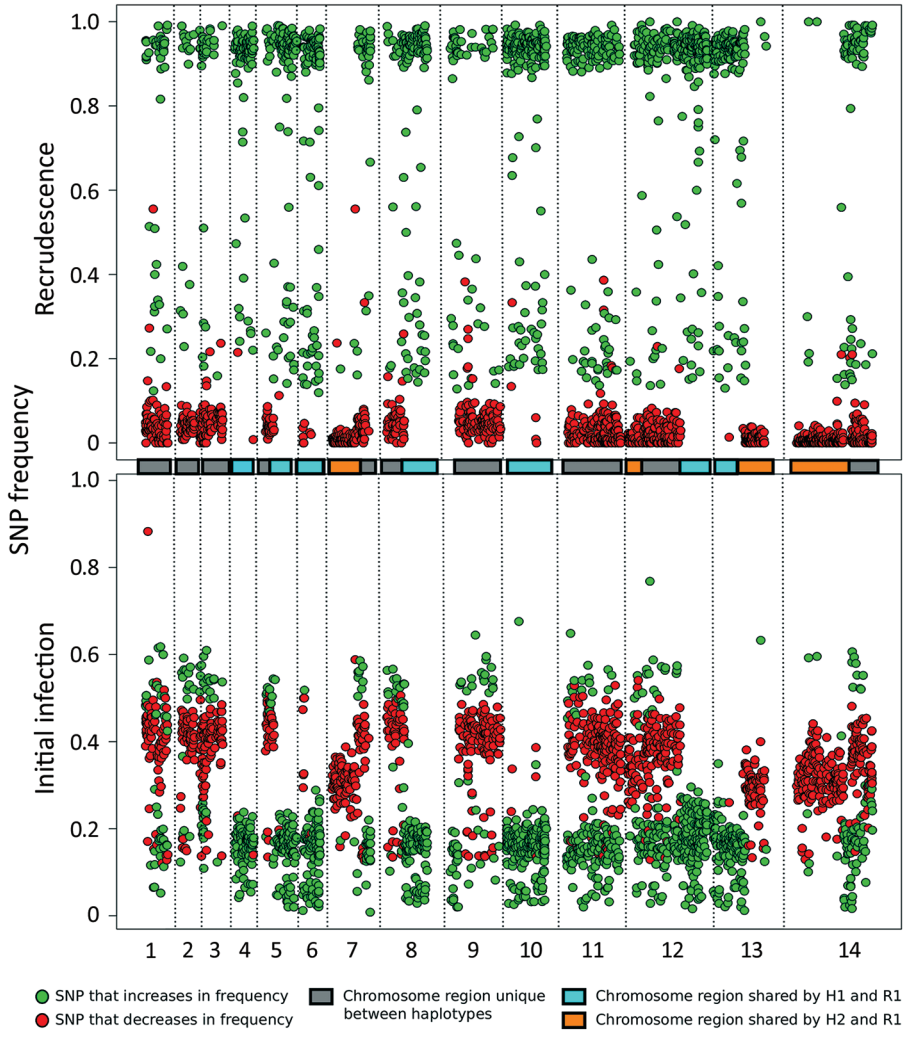

Figure 2. Analysis of the minor haplotype (H2) that caused recrudescence of *Plasmodium malariae* infection in a patient at Royal Darwin Hospital, Darwin, Northern Territory, Australia, March–April 2015, showing distribution of SNP alternative (nonreference) allele frequencies across the 14 chromosomes (boxes in the middle and dotted vertical lines) in the initial infection (bottom plot) and the recrudescence (top plot). The SNP colors (green, increasing in frequency; red, decreasing in frequency) form 2 clear bands, corresponding to H1 (yellow box) and H2 (pink box). H2 probably caused the recrudescence given that all of its alleles increase considerably in frequency. Colored boxes in center of chart indicate chromosome sharing. H1, haplotype 1; H2; haplotype 2; R1, reference genome; SNP, single-nucleotide polymorphism.

Table 6), probably because of the low coverage depth and because they were in repetitive regions.

Unexpectedly, several SNPs at high allele frequencies (>0.4) also increased in frequency in the recrudescent strain. Testing by using additional *P. malariae* samples (*15*) showed that ≈80% of these SNPs were homozygous for the alternate allele in ≥1 other *P. malariae* samples, whereas ≈30% were homozygous in all other *P. malariae* samples (online Technical Appendix Figure 5). This observation indicated that of these unusual SNPs, ≈50% were highly polymorphic, whereas ≈30% were probably low-frequency SNPs with rare variants present in the reference strain. This would explain the observation of SNPs with high reference allele frequency in the initial infection that became homozygous alternate in the recrudescence, given that they were probably SNPs with alternate alleles shared by H1 and H2.

To clarify the relationships of the different haplotypes with each other, we classified every genome region by whether any of the 3 haplotypes were identical to each other (Figure 2; online Technical Appendix). Approximately 25% of the genome is shared between H1 and R1 and between H2 and R1. No regions were shared between H1 and H2, which suggested that both H1 and H2 were half-siblings of R1, although they did not share any parent between themselves (online Technical Appendix Figure 6). The finding that all haplotypes were related to each other through R1 further suggested that all strains were transmitted from the same mosquito bite and that the mosquito ingested at least 4 different parental haplotypes (online Technical Appendix Figure 6).

Analysis of SNPs in orthologs of known drug-resistance genes identified 3 nonsynonymous SNPs in the multidrug resistance protein 2 (*mdr2*) gene, 1 of which was in the ABC transporter domain, and 2 in the ABC transporter domain of ABC transporter C family member 2, present in the recrudescent strain (H2) but not the other strains (online Technical Appendix Table 7). No evidence was found for copy number variation in any gene compared with the reference strain, and the reference strain did not appear to

have an amplification of the multidrug resistance protein 1 gene compared with any of the other *P. malariae* samples.

Discussion

This report of a case of recurrent *P. malariae* malaria is unusual in that it describes the molecular characterization and confirmation of a treatment failure after directly observed, appropriately administered, quality-assured AL dosing in a nonendemic environment where reinfection was not possible. Whole-genome sequencing demonstrated that the recrudescence was attributable to a minor clone present in the initial polyclonal infection. The case raises 2 important questions: first, what was the cause of treatment failure; and second, why did recrudescence arise from the minor clone rather than a dominant reference clone?

Although the efficacy of AL for *P. malariae* infection is assumed in many national guidelines (*18*), *P. malariae* monoinfections are relatively unusual and often of low density. To our knowledge, there have been no published efficacy series of AL with the long follow-up necessary to assess efficacy against a parasite with a 72-hour life cycle. In a nonrandomized efficacy study of 4 PCR-confirmed *P. malariae* infections treated with AL in Gabon (1 *P. malariae* monoinfection and 3 mixed *P. malariae/P. falciparum* infections), all 4 were microscopy negative at day 28, with no follow-up beyond this time (*19*). Among 80 PCR-confirmed *P. malariae/P. falciparum* mixed species infections in Uganda, 12% were still PCR-positive for *P. malariae* at day 7 and 6% were still PCR-positive on day 17 (*20*). An additional 3 reports have documented *P. malariae* infections occurring at 38 days, 47 days, and 4 months after AL treatment of an initial microscopy-diagnosed *P. falciparum* infection in returned travelers with no further possible malaria exposure (*21–23*).

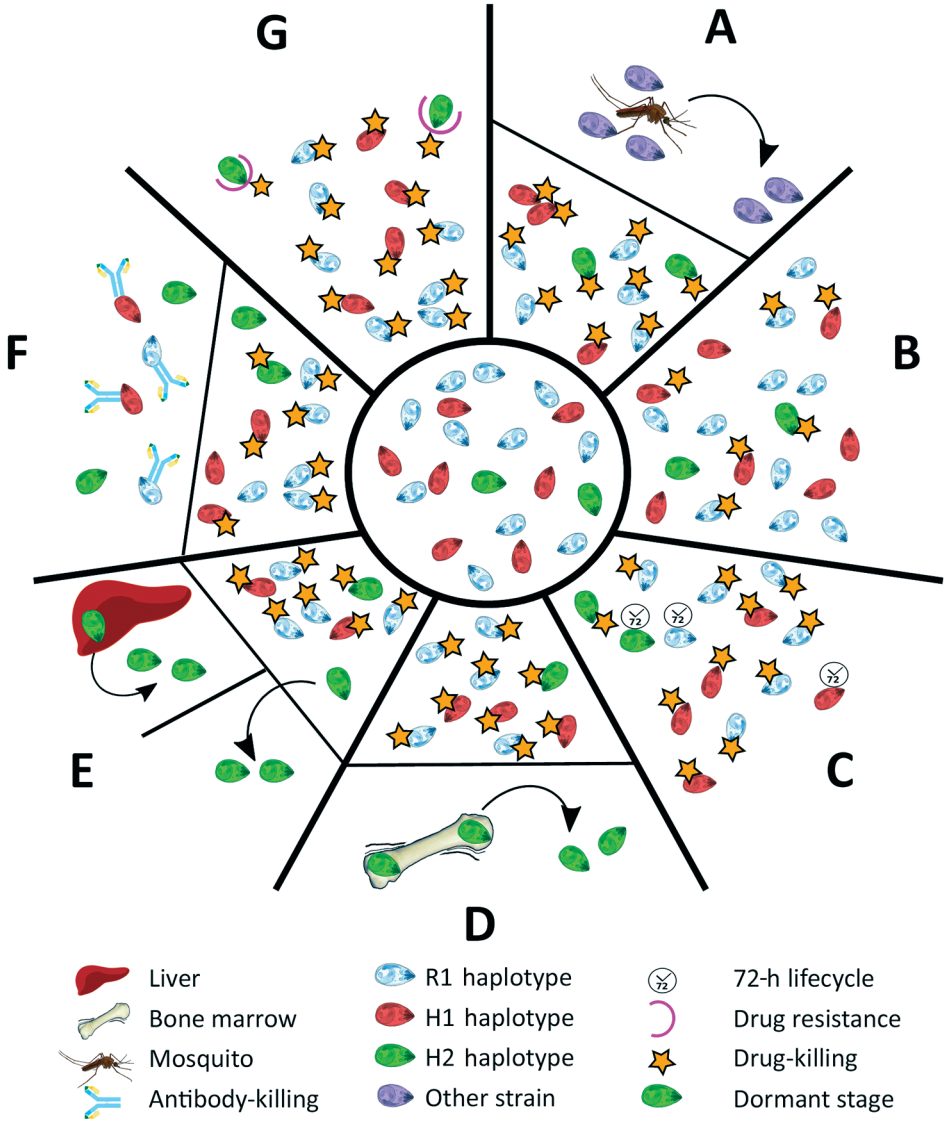

Figure 3. The different scenarios under which a second Plasmodium malariae infection could have occurred from the initial infection diagnosed in a patient at Royal Darwin Hospital, Darwin, Northern Territory, Australia, March–April 2015. Initial infection is shown in the inner circle. A) A completely new infection might have caused the second malaria onset. B) The drug might not have been absorbed at sufficient levels to kill all the parasites in the blood (pharmacokinetic cause). C) The longer intraerythrocytic life cycle of P. malariae (72 hours) might have enabled some parasites to survive the drug action until lumefantrine concentrations became subtherapeutic (pharmacokinetic cause). D) H2 parasites might have differentially sequestered with a biomass out of proportion with the peripheral parasitemia. E) Some parasites might have formed dormant stages in the liver, blood, or elsewhere (pharmacodynamic cause). F) An immune response might have been differentially primed against haplotypes at higher biomass. G) A haplotype within the initial infection might have been relatively drug resistant (fitness advantage). H1, haplotype 1; H2, haplotype 2; R1, reference genome.

Several plausible explanations might account for a recurrence of *P. malariae* parasitemia after treatment with AL (Figure 3). The last indigenous case of malaria in the Northern Territory was in 1962, with no subsequent cases of introduced malaria or autochthonous transmission (*24*); hence, the possibility of reinfection can be excluded (Figure 3, panel A). Additionally, the presence of the H2 haplotype in the initial infection and recrudescent infection confirms treatment failure.

Inadequate drug absorption resulting in suboptimal serum drug concentrations can cause treatment failure (Figure 3, panel B). Artemether is rapidly absorbed and eliminated (half-life of a few hours), whereas lumefantrine is variably absorbed and more slowly eliminated (half-life ≈3.2 days) (*25*). Lumefantrine is a lipophilic compound with erratic bioavailability unless administered with a small fatty meal (*26*), and for this reason, guidelines recommend administration of AL with a fatty meal such as milk or a small biscuit. In the case of our patient, we were unable to confirm adequate serum concentrations of lumefantrine; however, the patient took a complete course of treatment, and all doses were supervised in the hospital and administered with a milk biscuit to ensure good absorption. None of the treatment doses were vomited. In this scenario, one would expect >98% efficacy against *P. falciparum* (*27*). In addition, the clones associated with the R1 and H1 haplotypes, accounting for ≈90% of the parasite load, were cleared, suggesting that the plasma drug concentrations were sufficient to eliminate both infections. Nevertheless, considerable inter-individual variation exists in lumefantrine exposure, and this patient may have had relatively low concentrations.

Cure of malaria in a nonimmune patient requires that antimalarial blood concentrations are sustained above the parasites' MIC until the entire parasite biomass has been eliminated. In the presence of antimalarial drugs, the parasite biomass generally decreases over time in an exponential manner, with drug concentrations needing to be sustained above the MIC for ≥4 life cycles (*28*). In the case of our patient, the baseline parasitemia at initial presentation was 12,000/μL, which is relatively high compared with most *P. malariae* clinical infections (*6*). Thus, the combination of the long parasite life cycle such that 1 rather than 2 asexual cycles were exposed to artemether, and the short period (≈16 days) for which lumefantrine was at concentrations sufficient to kill the parasite may have resulted in parasites surviving the initial treatment and reestablishing a chronic parasitemia that was then sustained for 50 days before recrudescing (Figure 3, panel C).

Another possibility is that some parasites could have sequestered (Figure 3, panel D) or become dormant (Figure 3, panel E). Whereas dormancy would allow a proportion of the parasites to evade blood-stage schizontocidal activity, plausible sites for sequestration of *P. malariae*–infected erythrocytes would still be exposed to therapeutic concentrations of blood-stage antimalarials, making this possibility an unlikely explanation for this patient's recrudescent infection. *P. malariae* is well-recognized as having a prolonged preerythrocytic phase and a prepatent period of 16–59 days (*6*). The initial treatment course of AL was administered 56 days after the patient left Uganda, so any preerythrocytic stages were probably not present at the time of initial AL treatment.

Although the ability to form hypnozoites (dormant exoerythrocytic stages) occurs in 3 human malaria parasite species (*P. vivax*, *P. ovale curtisi*, and *P. o. wallikeri*), the evidence that latent exoerythrocytic stages do not occur in *P. malariae* is limited (*6*). Case reports have documented *P. malariae* producing symptomatic disease many years after exposure to infection, as noted in the case of a 74-year-old woman in Greece with *P. malariae* reactivation after ≥40 years (*29*). Such latency suggests that low-level parasitemia could persist for many years after infection, and indeed may be lifelong. In the case of our patient, parasite recrudescence occurred >100 days after he had left a malaria-endemic area.

Although inadequate drug absorption (Figure 3, panel B), duration of treatment (Figure 3, panel C), or dormancy (Figure 3, panel E) all may have contributed to parasite recrudescence, these indiscriminate explanations would be expected to occur primarily in the dominant strain during the initial infection (*28*) (online Technical Appendix Figure 7). One could speculate that the H2 minor parasite population might have emerged from a hepatic schizont that ruptured days after those giving rise to the majority haplotypes and, despite genetic similarity, had substantial differences in surface antigenicity. The antibody response to the primary infection, which would have reached a maximum ≈3 weeks after the illness began, would have been directed against the majority haplotypes and might not have recognized the minor population (Figure 3, panel F). Alternatively, more of the minor population might have been in the dormant state compared with the dominant circulating clones with R1 or H1 haplotypes, or more might have been at a higher biomass in erythrocytes sequestered elsewhere, enabling a proportion to evade antimalarial drug action and recrudesce.

Finally, the minority clone with H2 haplotype might have recrudesced because of a fitness advantage over the other clones/haplotypes (online Technical Appendix Figure 7, panels B, C), possibly including relative resistance to either artemether or lumefantrine (Figure 3, panel G). In *P. falciparum,* resistance to artemether is acquired through mutations in the propeller domain of *K13* (*30*), whereas *P. falciparum* resistance to lumefantrine is associated with mutations and copy number

variation in the *Pfmdr1* gene (*31*,*32*). Although neither of these genes had nonsynonymous mutations in H2, 1 nonsynonymous mutation was noted in the ABC transporter domain of *mdr2*, potentially involved in artemisinin resistance (*33*,*34*), and 2 nonsynonymous mutations were noted in the multidrug resistance–associated protein 2 gene, which has been implicated in reduced ex vivo susceptibility to lumefantrine in *P. falciparum* (*35*). We also identified 2 nonsynonymous SNPs in the dihydrofolate reductase homologue. Low serum concentrations, a modest reduction in lumefantrine efficacy, the prolonged life cycle of *P. malariae*, and rapid elimination of lumefantrine all might have contributed to the observed treatment failure in our patient.

In conclusion, we have described a case of *P. malariae* recrudescence occurring in a non–malaria-endemic country after adequately administered AL. Whole-genome sequencing data revealed that the monoclonal recrudescence consisted of a minor haplotype that accounted for ≈10% of the initial infection and that all the haplotypes in the initial infection were related to each other and therefore probably originated from the same infective mosquito bite. Although the haplotypes were closely related, the genomic data suggest that ≥4 parental haplotypes were ingested by the mosquito, indicating considerable diversity and transmission of *P. malariae.* This case raises concerns about the adequacy of ACTs with a short half-life partner drug, such as AL, in treating *P. malariae* infections and suggests that optimal ACTs to treat *P. malariae* should include a slowly eliminated partner drug. Our findings reinforce the importance of a longer duration of follow-up monitoring of patients infected with *P. malariae* for late recrudescence.

The sequencing data generated in this study are provided in the European Nucleotide Archive under accession codes ERS1110316 and ERS1110319.

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Author contributions: I.M., G.K.L.H., N.M.A., and R.P. oversaw the clinical presentation and management of the patient; S.A., and J.M. coordinated laboratory processing and collection of isolates; T.D.O. and R.N.P. designed the study; M.S. coordinated sequencing; G.G.R. performed the data analysis and with I.M. wrote the manuscript; and N.M.A., M.B., C.I.N., N.J.W., S.A., J.M., N.M.A., T.D.O., and R.N.P. critically revised the manuscript. All authors read and approved the manuscript. The authors declare that they have no conflicts of interest.

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Genomic Characterization of Recrudescent *Plasmodium malariae* after Treatment with Artemether/Lumefantrine

Technical Appendix

Supplementary Methods

Ethics Statement

The protocol used to collect human blood samples for patients with malaria attending Royal Darwin Hospital was approved by the Health Research Ethics Committee of Menzies School of Health Research (HREC 09/83). Written informed consent was obtained from the patient.

Sample Collection

Plasmodium malariae DNA used in this study was isolated from a symptomatic patient who presented to the Royal Darwin Hospital, Australia in March and April 2015 with a *P. malariae* parasitemia detected by blood film examination. At each episode, 5ml of EDTA blood was collected from the patient for routine confirmation of malaria by microscopy, full blood count, urea and electrolytes and liver function tests. An additional 10ml of EDTA blood was collected and leukodepleted by passage through a Plasmodipur filter (Euro-diagnostica) within 6 hours of collection. DNA was extracted from a 2ml aliquot of the filtered red blood cell pellet using the QIAamp DNA Blood Midi Kit (Qiagen) as per the manufacturer's instructions, and stored at –20°C. *Plasmodium* species was confirmed by PCR for *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale* parasites using a modified version of that described by Padley et al. (*1*) so that each species was identified in a separate (non-multiplex) assay. PCR for *P. knowlesi* parasites was undertaken using the method of Imwong et al. (*2*).

Genome Sequencing

Whole genome sequencing was performed on both parasite isolates (PmUG01 and PmUG02) using Illumina Standard libraries of 200–300bp fragments and amplification-free

libraries of 400–600bp fragments were prepared (*3*) and sequenced on the Illumina HiSeq 2000 v4, the MiSeq v2, and the X Ten according to the manufacturer's standard protocol. Raw sequence data were deposited in the European Nucleotide Archive (Technical Appendix Table 1).

Genotyping of Single Nucleotide Variants (SNPs)

The two *P. malariae* samples (PmUG01, PmUG02) were mapped against the *P. malariae* reference genome (*4*) using SMALT (-y 0.8, -i 500). The resulting bam files were merged, and GATK's (*5*) UnifiedGenotyper was used to call SNPs from the merged bam files (Technical Appendix Table 2). According to GATK's (*5*) best practices, SNPs were filtered by quality of depth (QD >2), depth of coverage (DP >20), mapping quality (MQ >30), and strand bias (FS <60). SNPs in low-complexity regions, as determined by Dustmasker (*6*), were removed, as were sites with missing data in either of the two samples, and SNPs within 50bp of each other to avoid SNPs in repetitive regions. Finally, only exonic SNPs were retained. We performed the same SNP calling procedure by also including additional previously published samples (*4*). Heterozygous sites were filtered out, while SNPs in non-coding regions were retained (Technical Appendix Table 3). Raw SNPs for PmUG01 and PmUG02 differ between Technical Appendix Tables 2 and 3 due to calling SNPs from a merged bam file instead of individually. Samples are therefore pooled and SNP calling is performed on the population rather than on the individual. The consequence of this is that if an individual sample has insufficient reads at a particular locus to reliably call a SNP there, that SNP might still be called if other samples in the population also have SNPs at that location, as this increases the likelihood of a SNP at that position. The number of SNPs therefore differs for identical samples depending on the population on which the SNP calling was performed.

Abundance Calculations

Using the relative SNP frequencies of the three haplotypes (R1, H1 and H2), the relative abundances of the different haplotypes were calculated, assuming that the number of sequencing reads is proportional to the abundance of the specific haplotype in the blood. From the SNP frequencies, H1 and R1 are in a ratio of 0.35:0.65 and H2 to R1 is in a ratio of 0.15:0.85. In both cases we ignore the third haplotype because we cannot ascertain its genotype. Ratio multiplication yields a joint ratio of 975:2975:5525 for H2:H1:R1, simplifying to ≈10:30:60. Tri-allelic sites offer the most straightforward way of observing the ratio of the three haplotypes,

however their number is low. Following SNP filtering (see above), the retained 13 tri-allelic sites were spread evenly across the genome (Technical Appendix Table 5). Assuming that the allele with the highest depth is R1, the intermediate depth is H1, and lowest depth is H2, we calculated the mean depth for all three. This yielded a ratio of ≈9:22:69 for H2:H1:R1 (Technical Appendix Table 5).

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Technical Appendix Table 1. Sequencing Information and Statistics

Characteristic	PmUG01	PmUG02
Accession Number	ERS1110316	ERS1110319
Origin	Uganda	Uganda
Infection	Initial	Recrudescence
Mean Coverage Depth	407x	136x
Coverage Range (Min-Max)	0x-7499x	0x-3519x
% Genome Covered at 1x	99.9%	99.9%
Sequencing Platform	Illumina HiSeq X Ten Illumina MiSeq v2 Illumina HiSeq 2000 v4	Illumina HiSeq X Ten Illumina MiSeq v2 Illumina HiSeq 2000 v4
Library Type	Amplification Free	Amplification Free

Technical Appendix Table 2. SNP Calling Results specifically for both PmUG01 and PmUG02

Sample ID	PmUG01	PmUG02
Raw SNPs	274,494	287,610
Private	79,953	93,069
Ref	362,848	333,615
Missing*	298	1,470
Filtered SNPs	2,442	1,499
Private	1,132	189
Ref	189	1,773
Missing*	0	0

*Sites at which the sample has no coverage. SNP calling results as per mapping the two *P. malariae* samples from the present clinical case against the PmUG01 reference genome (*4*). The raw SNPs are the total number of SNPs that we called using GATK's UnifiedGenotyper default parameters in the different samples. Of these raw SNPs, some are exclusive to a certain sample (Private), are identical to the reference genome (Ref), or there is no coverage and therefore no SNP call could be made (Missing). The same information is also shown for the filtered SNPs, which were filtered according to several different parameters (Methods).

Technical Appendix Table 3. SNP Calling Results for all *P. malariae* samples

Sample ID	PmUG01	PmUG02	PmMY01	PmID01	PmMA01	PmGN01
Origin	Uganda	Uganda	Malaysia	Papua Indonesia	Mali	Guinea
Raw SNPs	200,679	191,766	252,172	187,327	198,029	503,175
Private	13,300	10,462	48,632	19,396	26,637	73,208
Ref	531,180	479,142	367,672	347,257	360,155	398,393
Missing*	5,468	23,064	65,361	107,355	95,973	38,609
Filtered SNPs	1,375	2,707	22,696	17,564	16,057	21,329
Private	0	414	8,816	5,939	6,079	10,343
Ref	49,647	47,868	27,521	32,762	34,205	29,021
Missing*	0	0	0	0	0	0

*Sites at which the sample has no coverage. SNP calling results as per mapping all *P. malariae* samples against the PmUG01 reference genome (*4*). The raw SNPs are the total number of SNPs that we called using GATK's UnifiedGenotyper default parameters in the different samples. Of these raw SNPs, some are exclusive to a certain sample (Private), are identical to the reference genome (Ref), or there is no coverage and therefore no SNP call could be made (Missing). The same information is also shown for the filtered SNPs, which were filtered according to several different parameters (Methods).

Technical Appendix Table 4. Changes in genotype calls between the two infections

Initial Genotype	Recrudescence Genotype	Number of Sites
HR	HA	1
h	h	217
h	HR	1,178
h	HA	1,038
HA	h	1*
HR	h	196
HA	HR	0

HR = Homozygous reference HA = Homozygous alternate h = heterozygous
* in repetitive region of rhoptry-associated membrane antigen

Technical Appendix Table 5. Sequencing reads for the three haplotypes in tri-allelic sites in PmUG01

Genomic Location	H2 haplotype reads	H1 haplotype reads	R1 haplotype reads
Chr1: 704,003	6	21	202
Chr2: 437,291	43	71	130
Chr7: 1,580,127	12	18	75
Chr9: 1,095,695	39	91	117
Chr9: 1,429,882	21	82	140
Chr10: 873,165	9	31	209
Chr11: 987,107	17	60	173
Chr11: 2,535,419	47	84	113
Chr12: 2,746,074	9	44	192
Chr12: 2,855,999	11	36	176
Chr12: 3,047,435	11	34	204
Chr13: 1,061,473	13	16	221
Chr14: 1,118,313	27	62	139
Total (Proportion)	265 (0.09)	650 (0.22)	2,091 (0.69)

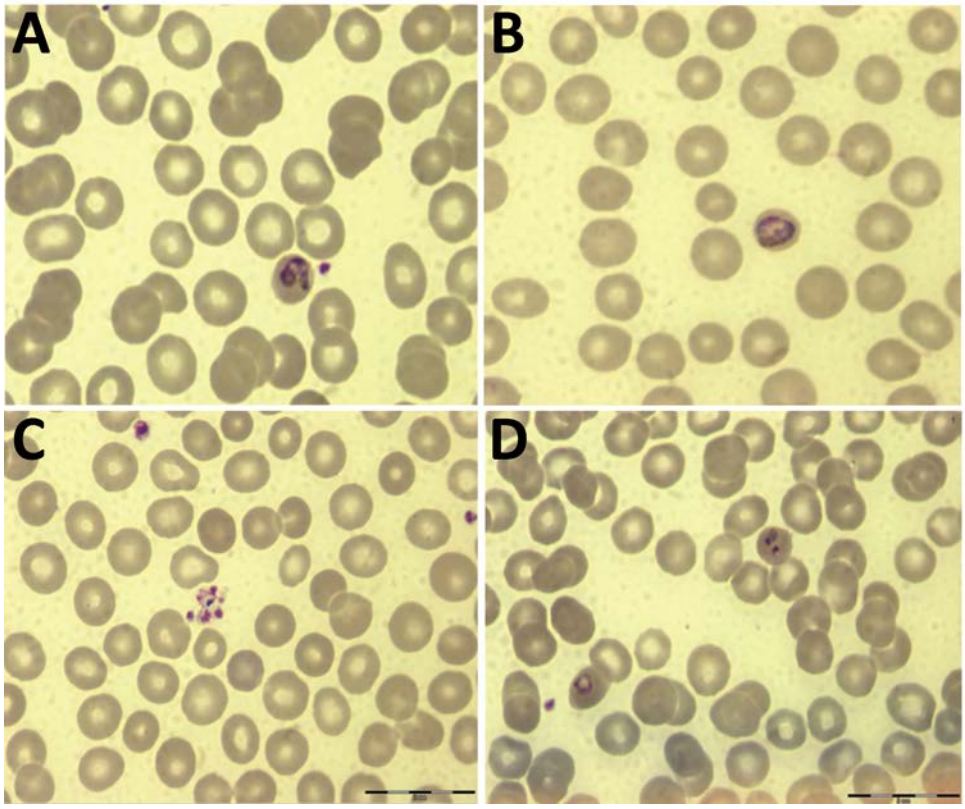
Assuming that the lower-level genotype is H2, intermediate-level genotype is H1, and higher-level genotype is R1, the tri-allelic sites have a certain number of sequencing reads confirming each of these three genotypes.

Technical Appendix Table 6. Sequencing reads for the three haplotypes in tri-allelic sites in PmUG02

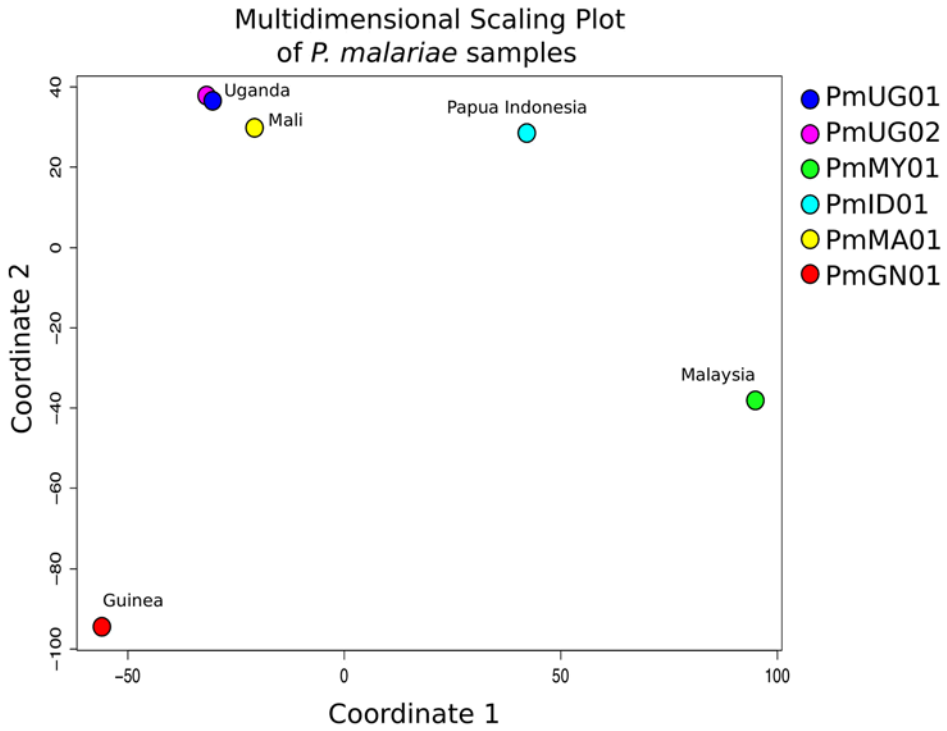
Genomic Location	H2 haplotype reads	H1 haplotype reads	R1 haplotype reads
Chr1: 704,003	16	0	7
Chr2: 437,291	20	4	0
Chr7: 1,580,127	18	0	9
Chr9: 1,095,695	25	0	0
Chr9: 1,429,882	23	0	0
Chr10: 873,165	19	8	1
Chr11: 987,107	11	0	7
Chr11: 2,535,419	15	0	1
Chr12: 2,746,074	9	0	1
Chr12: 2,855,999	6	4	4
Chr12: 3,047,435	16	0	0
Chr13: 1,061,473	8	0	5
Chr14: 1,118,313	26	0	0
Total (Proportion)	212 (0.81)	16 (0.06)	35 (0.13)

Technical Appendix Table 7. H2 specific nonsynonymous and synonymous mutations in drug resistance genes

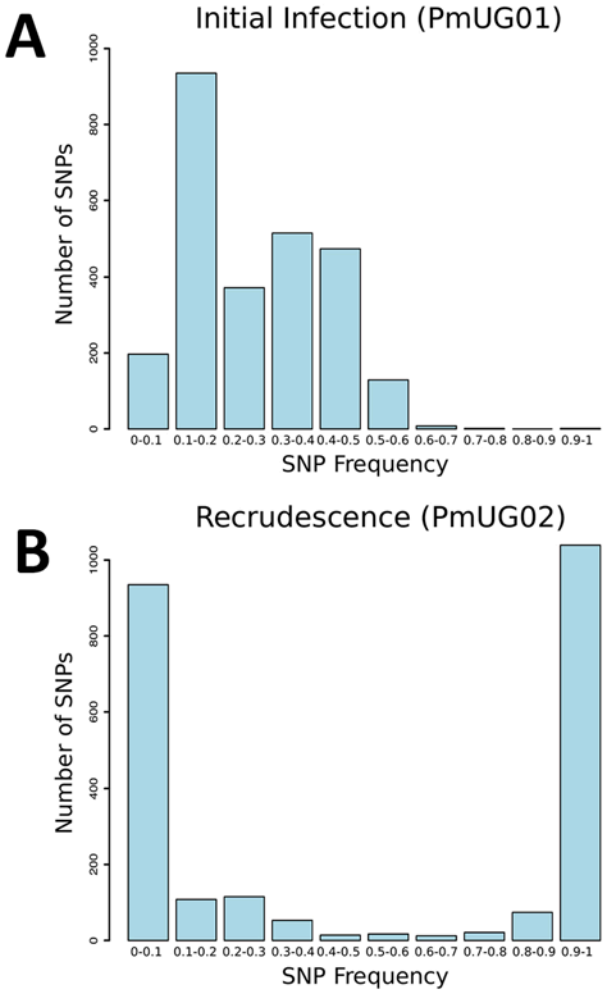
Gene ID (PmUG01_*)	Gene Description	*P. falciparum* ortholog	nsSNPs	sSNPs	Resistance
01020700	Chloroquine resistance transporter	0709000	0	0	Chloroquine (*8*), Quinine
10021600	Multidrug resistance protein 1	0523000	0	2	Multiple
12069100	Multidrug resistance protein 2	1447900	3	0	Multiple, Artemisinin (*9*)
14053100	AP-3 complex subunit mu	1218300	0	0	Artemisinin (*10*,*11*)
02019300	Ubiquitin carboxyl- terminal hydrolase 1	0104300	0	0	Artemisinin (*11*)
02011900	ABC transporter C family member 1	0112200	0	0	Artemisinin (*12*), Sulfadoxine/Pyrimeth amine (*13*)
14063400	ABC transporter C family member 2	1229100	2	0	Chloroquine (*14*), Piperaquine (*14*), Mefloquine (*14*) Lumefantrine (*15*)
02017400	Calcium-transporting ATPase	0106300	0	0	Artemisinin (*16*)
13021900	P-type ATPase4	1211900	0	1	Spiroindolones (*17*), Pyrazoleamides (*18*), Dihydroisoquinolone s (*19*)
12021200	Kelch protein k13	1343700	0	0	Artemisinin (*20*)
14020100	Sodium/hydrogen exchanger 1	1303500	0	0	Quinine (*21*)
05034700	Bifunctional dihydrofolate reductase- thymidylate	0417200	2	1	Pyrimethamine (*22*)
14036800	ATP-dependent Clp protease adaptor protein	0810800	0	0	Sulfadoxine (*23*)
MIT001100	Cytochrome b	mal_mito_3	0	0	Atovaquone (*24*)



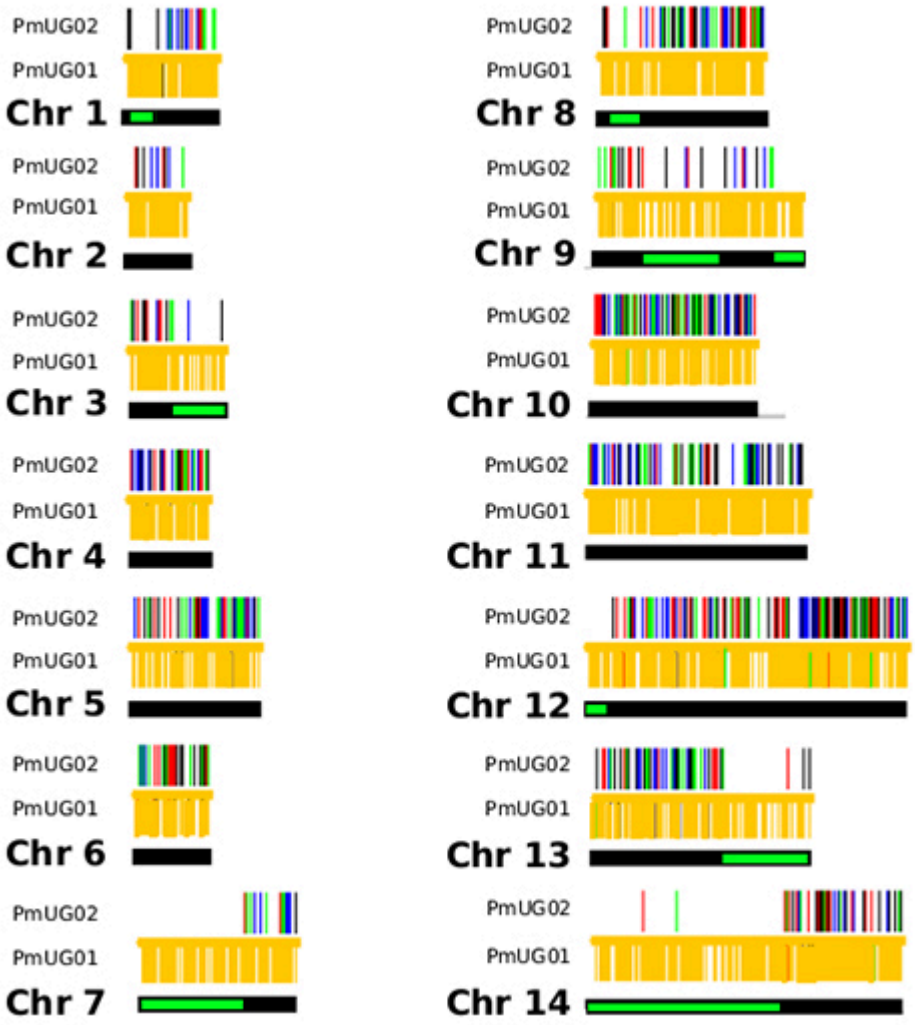
Technical Appendix Figure 1. Positive *Plasmodium malariae* thin blood films. Thin smear scans of the initial infection (A, B) and of the recrudescence (C, D), both indicating a *P. malariae* infection.



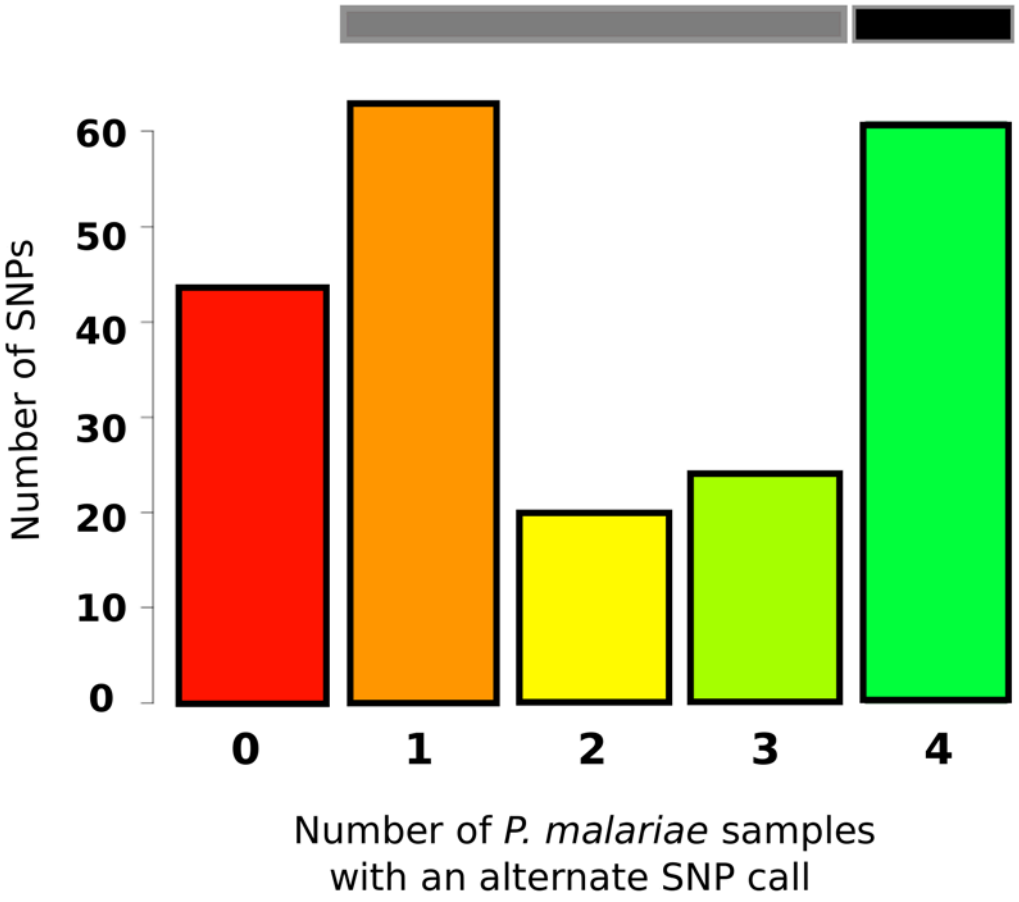
Technical Appendix Figure 2. Similar SNP spectra for PmUG01 and PmUG02. Multidimensional scaling plot based on differences in SNP spectra between the different *P. malariae* samples (*4*), showing that the initial (PmUG01) and the recrudescent (PmUG02) infections are significantly more similar to each other than to the other *P. malariae* samples. This suggests that the two infections have a similar origin.



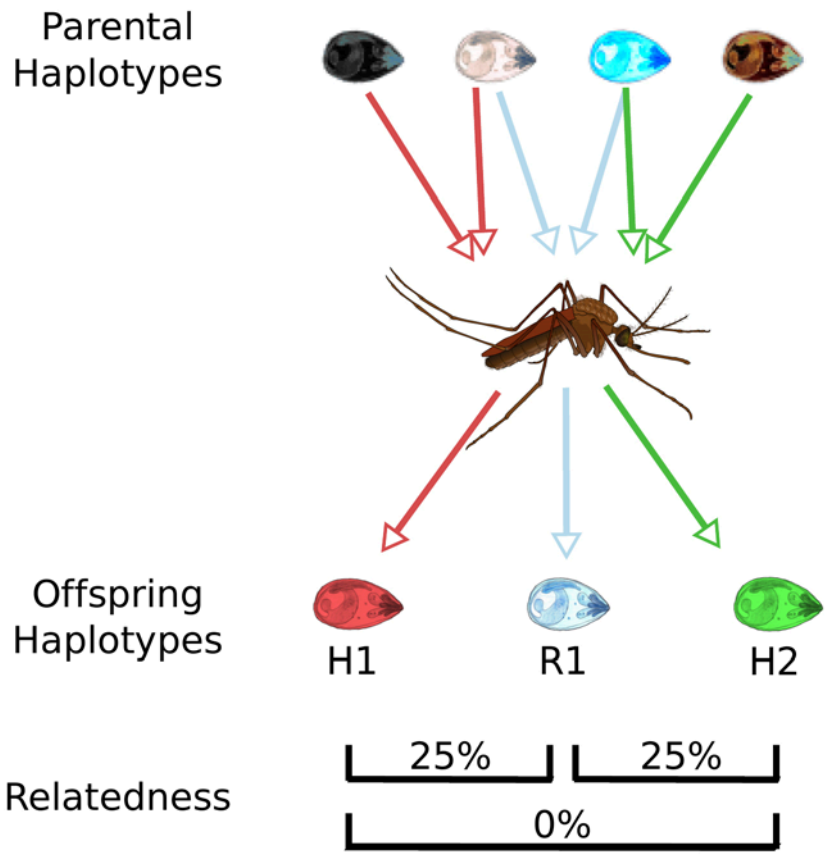
Technical Appendix Figure 3. Differences in SNP frequencies between PmUG01 and PmUG02. SNP frequency bar plots for both the initial infection (A) and the recrudescence (B), showing that there was a significant shift in the SNP frequency spectra between the two infections, with the initial infection being a polyclonal infection, while the recrudescence seems to be monoclonal. Interestingly, the initial infection seems to have a bimodal distribution of heterozygous SNPs.



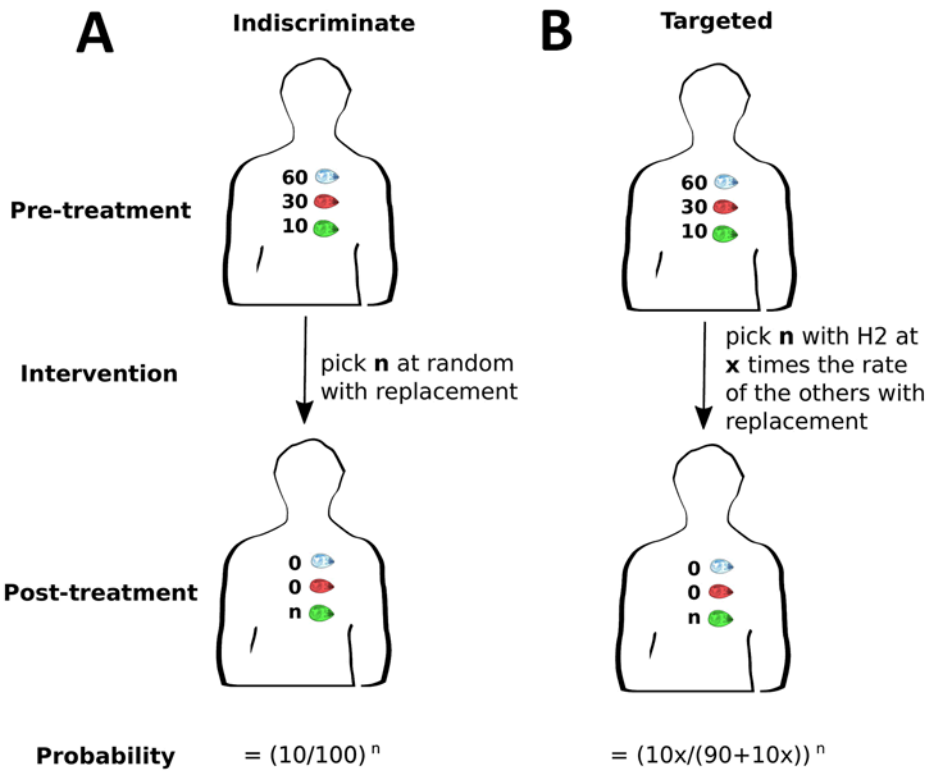
Technical Appendix Figure 4. Clustered SNP distribution in PmUG02 across chromosomes. Distribution of heterozygous sites (yellow) in the initial infection (PmUG01) and homozygous alternate sites (other colors) in the recrudescence (PmUG02) across the 14 chromosomes of *P. malariae*. The different colors for the homozygous alternate SNPs are arbitrary. Chromosome regions with more heterozygous sites in the initial infection becoming homozygous reference than becoming homozygous alternate in the recrudescence are marked in green. Genotypes were plotted using Artemis (*7*).

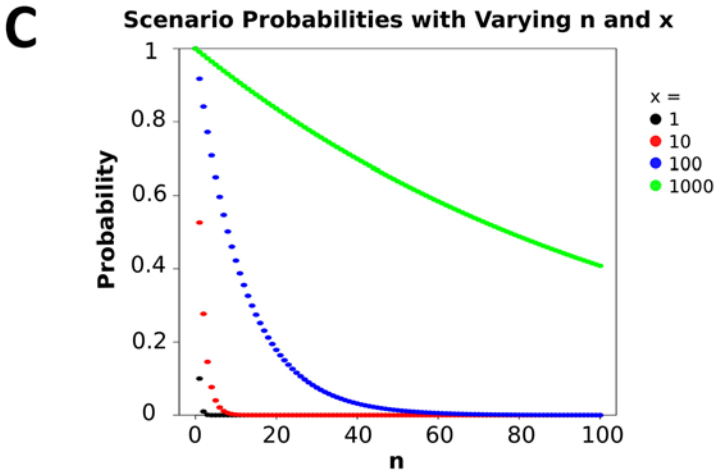


Technical Appendix Figure 5. Unusual SNPs in other *P. malariae* samples. Presence/absence in other *P. malariae* samples of SNPs with SNP frequencies of over 0.4 in the initial infection that increased in frequency in the recrudescence. The black bar indicates SNPs that are present in all other *P. malariae*, suggesting that the reference strain is rather the variant compared to the general population. The gray bar indicates SNPs that are present in multiple other *P. malariae* samples, suggesting that they are highly polymorphic sites. In sum, this suggests that most of these SNPs are likely SNPs shared by both H1 and H2, explaining why they have high frequencies in the initial infection and in the recrudescence.



Technical Appendix Figure 6. Inferred number of parental haplotypes. The relatedness of the three haplotypes in the initial infection (offspring haplotypes) as inferred by the sharing of genomic regions. This sharing suggests that there were four parental haplotypes present in the mosquito that interbred to form the three haplotypes we see in the initial infection. Of these, it seems that R1 is a half-sibling with both H1 and H2, but via a different parental haplotype.





Technical Appendix Figure 7. Indiscriminate versus targeted interventions. Differences in probabilities for (A) indiscriminate interventions (i.e., explanations for a recrudescence) that affect all haplotypes in the infection equally, such as insufficient drug dosage or drug avoidance through a longer lifecycle, versus (B) targeted interventions that potentially affect one haplotype different to another, for example haplotype-specific drug resistance or an increased propensity for greater sequestered biomass in one haplotype. The two scenarios show that the difference is in how an intervention 'selects' for n number of parasites in the recrudescence. In an indiscriminate intervention, all haplotypes have the same probability of being

selected, while H2 is x times more likely to be selected in a targeted intervention. An indiscriminate intervention has a low probability (<0.05) at all values of n > 1 (C), while the probability of a targeted intervention increases across all values of n the higher x. If an indiscriminate intervention were to be the sole explanation for a recrudescence, then it would suggest that only a singly parasite survived from the entire initial infection, an unlikely scenario. On the other hand, a targeted intervention presents a more parsimonious explanation for the lower level haplotype recrudescing.